The role of the NK-homeobox gene slouch (S59) in somatic muscle patterning

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SUMMARY

In the Drosophila embryo, a distinct class of myoblasts, designated as muscle founders, prefigures the mature pattern of somatic body wall muscles. Each founder cell appears to be instrumental in generating a single larval muscle with a defined identity. The NK homeobox gene S59 was the first of a growing number of proposed ‘identity genes’ that have been found to be expressed in stereotyped patterns in specific subsets of muscle founders and their progenitor cells and are thought to control their developmental fates. In the present study, we describe the effects of gain- and loss-of-function experiments with S59. We find that a null mutation in the gene encoding S59, which we have named slouch (slou), disrupts the development of all muscles that are derived from S59-expressing founder cells. The observed phenotypes upon mutation and ectopic expression of slouch include transformations of founder cell fates, thus confirming that slouch (S59) functions as an identity gene in muscle development. These fate transformations occur between sibling founder cells as well as between neighboring founders that are not lineage-related. In the latter case, we show that slouch (S59) activity is required cell-autonomously to repress the expression of ladybird (lb) homeobox genes, thereby preventing specification along the lb pathway. Together, these findings provide new insights into the regulatory interactions that establish the somatic muscle pattern.

Key words: slouch, Drosophila, Myoblast, Homeobox, ladybird

INTRODUCTION

Myogenesis and muscle diversification are two intimately linked processes that lead to the formation of ∼30 distinct muscle fibers in each hemisegment of the Drosophila larva. Each of these muscles consists of a single syncytium and displays unique features with respect to its size, shape, epidermal attachments and innervation. Research in the past decade has provided significant insights into the development and patterning of the somatic musculature. An important advance has been the finding that each of the larval muscles is prefigured by a single mesodermal cell, termed muscle founder. Founder cells appear to seed muscle formation by fusing with surrounding myoblasts, termed fusion competent cells. Muscle founders were originally identified morphologically and through the specific expression of the S59 homeobox gene in a subset of these cells (Bate, 1990; Dohrmann et al., 1990). In addition, the analysis of muscle development in a fusion-defective mutant, myoblast city (mbc), strengthened the notion that it is the founder cells that assume defined identities during early mesoderm development (Rushton et al., 1995). By contrast, the fusion competent cells appear to be in a more plastic developmental state and become entrained into particular developmental programs only upon their fusion with specified founder cells. Thus, in order to explain the regulation of muscle patterning, it is pivotal to understand how each of the muscle founders develops and becomes specified. Interestingly, there exist striking similarities between muscle founder formation and early neuronal development in Drosophila. For example, it has been documented that each founder cell is derived from a progenitor cell, which divides mitotically to yield either two non-identical founders or, in some cases, one founder and one precursor cell of adult muscles (Dohrmann et al., 1990; Carmena et al., 1995). As neuroblasts during early neurogenesis, muscle progenitors are initially singled out from larger ‘promuscular’ cell clusters that are marked by the expression of the proneural gene lethal of scute (l’sc) (Carmena et al., 1995). Segregation of muscle progenitors from these clusters involves lateral inhibition processes that are mediated by the functions of neurogenic genes, including Notch, Delta and E(spl) (Corbin et al., 1991; Bate et al., 1993; Baker and Schubiger, 1996).

While a few expression markers, including lacZ in the enhancer trap line rP298 and myoDnautilus (Michelson et al., 1990; Paterson et al., 1991; Nose et al., 1998), are shared by most if not all muscle founders, the much more restricted expression of S59 suggested that muscle progenitors and founders are born with different identities. Specifically, S59 expression is observed, at least transiently, in the progenitors and founders of the ventral muscles 25 (VT1), 26 (VA1), 27 (VA2), 29 (VA3), a ventral adult muscle precursor, the lateral muscle 5 (LO1), the dorsolateral muscles 18 (DT1) and probably 11 (DO3), and then maintained only in muscles 18, 25 and 27 (Dohrmann et al., 1990; Bate et al., 1993; Carmena...
et al., 1995). Similarly restricted patterns of expression that include overlapping or different sets of muscle founders have also been described for several other putative or known transcription factors, including apterous (ap; Bourgoin et al., 1992), vestigial (vg; Bate et al., 1993), Krüppel (Kr; Ruiz-Gomez et al., 1997), even-skipped (eve; Frasch et al., 1987), msh (Lord et al., 1995; D’Alessio and Frasch, 1996; Nose et al., 1998), ptx-1 (Vorbrüggen et al., 1997), ladybird (lb; Jagla et al., 1998) and collie (coe; Crozatier and Vincent, 1999). The differential specification of muscle progenitors appears to be dictated partly by the differential activation of resident EGF and FGF receptors (Flb and Htl, respectively; Buff et al., 1998; Michelson et al., 1998). An additional source for muscle diversification is the asymmetric division of muscle progenitors, which generates two founder cells with different identities. This process is mediated by the asymmetric segregation of determinants such as Inscuteable and Numb within the dividing progenitors, which was shown to cause the differential maintenance of the expression of several of the aforementioned transcription factors in the two daughter cells (Ruiz-Gomez and Bate, 1997; Carmena et al., 1998b; Jagla et al., 1998; Park et al., 1998; Crozatier and Vincent, 1999).

There is mounting evidence that founder cell identities are determined by the expression of defined combinations of regulatory factors. Notably, loss of function and ectopic expression of ap, Kr, msh and lb can cause transformations in muscle founder identities and aberrant muscle patterns, while loss of nau or coe activity results in arrested differentiation of specific founder cells and absence of the particular muscles that they would normally form (Bourgoin et al., 1992; Ruiz-Gomez et al., 1997; Nose et al., 1998; Jagla et al., 1998; Keller et al., 1998; Crozatier and Vincent, 1999). For a more complete understanding of muscle diversification, it is however necessary to define the functions of additional identity genes, study the hierarchies of their genetic interactions and identify their regulatory targets.

Here we describe the genetic function of S59 in somatic muscle specification. We show that S59 is required for the normal development of all abdominal muscles that are derived from S59-expressing founder cells. Interestingly, loss of S59 activity has different effects in individual muscle types. For example, muscles 5, 25 and 29 are transformed into muscles (or precursors) with different identities, whereas muscles 18 and 27 are formed, albeit with aberrant features. We provide a detailed analysis of the role of S59 in the development of muscles 5 and 25, both of which are derived from a common muscle progenitor. We further show that S59 is required for the repression of lb in these cells to prevent them from developing into segment border muscles and lateral adult muscle precursors.

MATERIALS AND METHODS

Point mutation detection

To narrow down regions in the slou (S59) gene from slou285 that contain sequence alterations, individual exon fragments were PCR amplified from single embryos carrying the mutant chromosomes or paternal rutipa control versions, respectively. Selection of homozygous mutant embryos from a line balanced with a lacZ-expressing TM3 chromosome was based upon negative staining for S59 transcript (in muscles 25 and 27) and β-Gal. The generated fragments were endlabeled radioactively, the corresponding wild-type and mutant exon DNAs mixed in equimolar amounts, and then subjected to chemical cleavage as described in Saleeba and Cotton (1993). PCR with single mutant embryos was carried out as follows. After staining for S59 transcript and β-Gal activity, single embryos were selected and transferred into 10 μl ‘squishing’ buffer (Gloor and Engels, 1992) and digested with proteinase K, which was then heat inactivated at 95°C for 2 minutes. 2 μl of a total of 10 μl of the homogenized embryos were used in a 50 μl standard PCR reaction. After mismatch detection and subcloning, inserts from three independent clones were sequenced and showed a single, identical base pair change.

Histochemistry

Immunological stainings of whole-mount embryos, using the Vectastain ABC elite kit (Vector Laboratories) were done as described in Azpiazu et al. (1996).

The following primary antibodies were used: rabbit anti-S59 (Dohrmann et al., 1990), mouse anti-Lb monoclonal (Jagla et al., 1997), guinea pig anti-Krüppel (Kosman et al., 1998), rabbit anti-Twist (Roth et al., 1989), mouse anti-β-Gal (Sigma), rabbit anti-β-Gal (Cappel), mouse anti-Mysosin Heavy Chain (D. Kiehart, unpublished), rabbit anti-Nautilus (B. M. Paterson; Paterson et al. 1991). For S59 and Nau antibody stainings, Renaissance Tyramide Signal Amplification (TSA, NEN) was used in conjunction with the Vectastain ABC components. In the direct protocol, a 20 minute reaction with TSA fluorescent substrates was performed directly after binding of the ABC complex and standard washes. In the indirect protocol, a reaction with biotinyl tyramide was performed at this step, followed by a second incubation with ABC complex and finally a color reaction with fluorescein tyramide, Cy3 tyramide or dianimobenzidine (DAB).

Double fluorescent in situ hybridization and antibody labeling

Embryos where first hybridized following standard procedures except that proteinase treatment was omitted. After hybridization and washing, the embryos were incubated with sheep anti-digoxigenin antibody (Boehringer) for 1 hour followed by a 1 hour incubation with biotinylated goat anti-sheep antibody (Jackson). Subsequently, embryos were incubated with the ABC reagent. For fluorescent labeling, embryos were incubated for 20 minutes in 100 μl of a 1:50 dilution of tyramide substrate (Cy3 or fluorescein-conjugated, NEN) in amplification diluent. After 3× 30 minute washing in PBS (0.1% Tween 80) and HRP inactivation for 10 minutes at 70°C, the embryos were incubated with the primary antibody overnight at 4°C and for 1 hour at room temperature with the secondary antibody. Secondary antibodies either coupled with the fluorochrome or HRP were used. For HRP-coupled secondary antibodies, the TSA protocol was used as described above without the use of the ABC reagent. Fluorescent stainings were analyzed and processed with a Leica laser scanning microscope.

Histology

After removing heads and legs, adult flies were fixed in 4% formaldehyde in PBS overnight. After several washes, the samples were dehydrated through an ethanol series and infiltrated with parablast embedding medium (Fisher). 7 μm sections were cut using a HM 320 rotary microtome (Microm). The sections were dried and stained with Hematoxylin and Eosin using standard procedures.

Fly strains

The slou285 mutation was induced with EMS on a rutipa (ru th st ri p6 cau)-marked chromosome (Azpiazu and Frasch, 1993). For UAS-S59, the S59/2 cDNA (Dohrmann et al., 1990) was cloned into pUAST (Brand and Perrimon, 1993). Ectopic expression was achieved by

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activity and ectopic expression of slou

Muscle pattern defects upon loss of slou was renamed slouch. escapers, which are very weak, unable to reproduce and have a short life span, the favorable conditions. Based upon the phenotype of percentage (~10%) survive to adulthood under different stages of development, a small muscle myosin heavy chain (MHC). As shown in embryos was visualized with antibodies against the syncytial fibers at the normal positions of muscles 26 and 27 have aberrant and variable morphologies (Fig. 2A, arrows) although their insertions sites and sizes are still reminiscent of those two muscles. Whether they form two separate or one large syncytium in each segment could not be determined. Disruptions were also observed in lateral and dorsolateral areas of the body wall musculature. Specifically, muscle 5 is absent, whereas muscle 8 appears duplicated in each of the segments (Fig. 2B,E). At the normal positions of muscles 11 and 18, there is one syncytium (or perhaps two closely associated ones) with a morphology that does not resemble either of these two dorsolateral muscles, although it shares their ventral attachment site (Fig. 2B). In contrast to the altered ventral and dorsolateral muscle patterns, the pattern of the dorsal somatic muscles is normal in the absence of slou (S59) activity. This is consistent with the observed absence of S59 expression in these areas in wild-type embryos. Together, these observations show that loss of slou activity affects the development of all muscles derived from S59-expressing muscle founders. slou function appears to be strictly required in these muscles, because loss of muscles 5, 25, 29 and abnormalities associated with muscles 11/18 and 26/27 were observed in nearly 100% of all examined abdominal segments from mutant embryos. Whether these observed aberrations reflect cell fate transformations or abnormal differentiation will be discussed later.

Ectopic expression of slou (S59) in the mesoderm of wild-type embryos (see Materials and Methods) also produced severe alterations in the muscle pattern. As shown in Fig. 2G,H, the patterns of the ventral, lateral and dorsal somatic muscles are severely disrupted in these embryos, thus making it difficult to assign specific identities to individual muscle fibers. We also observe morphological changes in the dorsal vessel, where some of the cardioblasts appear enlarged or are arranged in clumps instead of single rows and express higher than normal levels of myosin. These observations indicate that expression of S59 in muscle founders and muscles where it is not normally expressed causes abnormal development and perhaps transformations of their identities (see below). The

RESULTS

Identification of a null mutation in S59

To generate mutations in the S59 gene, which maps to the previously described homeobox gene cluster at 93DE, Df(3R) e-D7 (93C3-6; 93F6-8) was saturated for lethal mutations by EMS mutagenesis (Azpiazu and Frasch, 1993; Azpiazu, 1994). Based upon its map position between the distal breakpoints of Df(3R)e-F1 and Df(3R)e-D7 as well as its associated muscle phenotype, a complementation group with a single hit appeared to be a good candidate for S59. Heteroduplex analysis for mismatches and sequencing of genomic DNA derived from homozygous mutant embryos (see Materials and Methods) identified a C-to-T transition, corresponding to position 983 of the previously reported S59/2 cDNA sequence (Dohrmann et al., 1990). This base change introduces a stop codon after amino acid 219 and truncates the protein by about two-thirds (Fig. 1). As expected, antibodies produced against a polypeptide from residues 188 to 659 failed to detect any S59 protein in homozygous mutant embryos. Together, these data suggest strongly that this allele, S59206, is a functional null. While the majority of animals that are homozygous for this mutation or transheterozygous over a deficiency die during different stages of development, a small percentage (~10%) survive to adulthood under favorable conditions. Based upon the phenotype of these escapers, which are very weak, unable to reproduce and have a short life span, the S59 gene was renamed slouch (slou).

Muscle pattern defects upon loss of activity and ectopic expression of slou (S59)

To assess the role of slou (S59) in muscle development, the muscle pattern in slou mutant embryos was visualized with antibodies against muscle myosin heavy chain (MHC). As shown in Fig. 2A,D, the pattern of ventral muscles is severely disrupted in these embryos and, notably, there is a complete absence of muscles 25 and 29. In addition, the syncytial fibers at the normal positions of muscles 26 and 27 have aberrant and variable morphologies (Fig. 2A, arrows) although their insertion sites and sizes are still reminiscent of those two muscles. Whether they form two separate or one large syncytium in each segment could not be
gene mutants (Ruiz Gomez and Bate, 1997) provide good wild-type embryos. We observe that expression is maintained in muscles 18 and 27 in late-stage S59 undergo abnormal differentiation. For further analysis, we used these muscles are transformed or retain their identities but 11/18 in identities (Keller et al., 1997).

partial transformations from cardial to somatic muscle cell, which were interpreted as upon ectopic expression of nau

Fig. 2. Somatic muscle phenotypes as a consequence of slou (S59) mutation and ectopic slou expression. Shown are flat preparations of body walls from embryos stained for muscle myosin heavy chain (MHC). In this and the following figures, dorsal is up and anterior to the left. (A) Muscles 25 and 29 are absent in slou mutant embryos (Their normal positions are indicated with a small (25) and large (29) asterisk). The syncytia occupying the normal positions of muscles 26 and 27 display aberrant and variable morphologies and insertion sites (arrows). (B) Muscle 5 is absent and muscle 8 is duplicated in each of the segments shown. Muscles 11/18 are abnormal. (C) The dorsal muscles in slou mutants appear normal. (D-F) Respective ventral, dorsolateral and dorsal somatic muscle groups from wild-type control embryos. Dv, dorsal vessel. (G,H) Upon
ectopic expression of slou in the mesoderm, ventral (G) and dorsolateral (H) muscle patterns appear severely disrupted. (I) The dorsal muscles display fewer disruptions upon ectopic slou expression, although some muscles also have aberrant shapes and insertion sites (arrow). In the dorsal vessel, many cardioblasts have irregular shapes and arrangements and display increased levels of MHC expression (arrowheads).

effects in the cardioblasts are reminiscent of those observed upon ectopic expression of nau, which were interpreted as partial transformations from cardial to somatic muscle cell identities (Keller et al., 1997).

Initial morphological examination of muscles 26/27 and 11/18 in slou mutants was not sufficient to determine whether these muscles are transformed or retain their identities but undergo abnormal differentiation. For further analysis, we used S59 as a marker for these two muscle types because its expression is maintained in muscles 18 and 27 in late-stage wild-type embryos. We observe that S59 mRNA or lacZ driven by an S59/lacZ construct, which mimics the normal pattern of S59 expression (see Materials and Methods), is still expressed in the founders of the morphologically aberrant muscles 11/18 and 26/27 in the slou mutant background (Fig. 3A,B). A second marker for muscle 27 is Kr, which has an important role in distinguishing the identity of this muscle from that of muscle 26 (Ruiz-Gomez et al., 1997). In wild-type embryos, Kr is coexpressed with S9 in the progenitor and founders of muscles 26 and 27, and similar to S59, Kr expression is lost in muscle 26 and maintained only in muscle 27 (Fig. 3A,C; Ruiz-Gomez et al., 1997). (Lineages for S59-expressing muscle founders are given as described in Dohrmann et al. (1990) and refined by Carmena et al. (1995). While there is presently no formal proof for these lineage relationships, the data from S59 antibody and S59/lacZ stainings and genetic analysis of lineage gene mutants (Ruiz Gomez and Bate, 1997) provide good

support for sibling relationships between muscle founders 5 and 25, muscle founders 26 and 27, and muscle founder 29 and the ventral adult precursor cell, respectively. There is also some, albeit less clear, evidence for an origin of muscle founders 18 and 11 from a common progenitor.) In slou (S59) mutant embryos, both S59 and Kr expression are initiated normally in the 26/27 progenitors and founders (Fig. 3B). These data were further confirmed by probing for nascent S59 transcripts of Kr-stained wild-type and slou mutant embryos with an S59 intron probe, which showed similar S59 transcription patterns in the founders of muscles 11/18, 26 and 27, and ventral adult precursors in both genotypes (Fig. 3C,D; note, however, the absence of expression in muscle 5 and 25 founders in slou mutants, which is discussed later).

In contrast to the founder cell stage, during fusion the expression of S59/lacZ (Fig. 3F), S59 mRNA (data not shown) and S59 nascent transcripts (Fig. 3H) is abolished in most (~85%) of the muscle 26/27 precursors in slou mutant embryos. However, unlike S59, Kr expression is fully maintained in the aberrant 26/27 syncytia until late stages in slou mutants (Fig. 3F). Expression of nautilus (nau), another marker for muscle 27 (Fig. 3I), is also not affected in the absence of slou activity (Fig. 3I). In contrast to Kr and nau, the expression of apetrous/lacZ (ap/lacZ) in the founders and syncytia of muscle 27 of slou mutant embryos is absent (Fig. 3L, compare with wild type in Fig. 3K). Together, these observations indicate that, in the absence of slou activity, the
progenitors of muscles 26 and 27 are formed normally. Later, the resulting muscle fibers appear to assume features that are intermediate between muscles 26 and 27 because they maintain Kr and nau (as is normally observed in 27) but fail to activate a muscle 27-specific enhancer of ap and lose S59 (as is normally the case for 26). The expression of Connectin in these aberrant syncytia also shows that they maintain certain features that are typical of both muscles 26 and 27 (data not shown).

As shown in the late stage wild-type embryo in Fig. 3G, which was probed with an S59 antibody and S59 intron probe, it is not only passive diffusion of the S59 protein but also active initiation of S59 transcription that spread to the newly acquired nuclei during the formation of multinucleate syncytia. Previously, we had speculated that S59 autoregulation could be involved in this event (Dohrmann et al., 1990). However, the observation of nascent S59 transcripts in all nuclei of the aberrant muscle 11/18 syncytia and in the few remaining muscle 27 syncytia (Fig. 3H) in the absence of slou activity contradict this hypothesis. Rather, it appears that slou

**Fig. 3.** Expression of S59, Krüppel, Nautilus, and apterous-lacZ during muscle development of wild-type and slou mutant embryos. Shown are high magnification views of embryos analyzed by confocal microscopy, with wild-type embryos to the left and slou mutant embryos to the right. The color codes for individual probes are indicated on the left. Double labeling produces yellow signals. (A,B) Mid-stage 12 embryos stained with an S59 riboprobe and Kr antibodies. In slou, Kr expression in all founders and S59 mRNA expression in muscle founders 18, 26 and 27 is normal, while S59 signals in muscle 5 founders are missing (neither wild-type nor slou mutant embryos express S59 mRNA in muscle founder 25 at this stage). (C,D) Mid-stage 12 embryos stained with an S59 intron probe and Kr antibodies, confirming the normal pattern of S59 transcription in muscle founders 18, 26 and 27, and ventral adult precursors (VaP) and the lack of S59 expression in muscle 5 founders in slou mutant backgrounds. (E,F) Stage 15 embryos carrying the RRHS59-lacZ reporter construct, probed with β-Gal and Kr antibodies. In the slou mutant embryo, only one of the segments shown displays S59/lacZ expression in muscle 27, although all segments show Kr expression in the corresponding syncytia. S59/lacZ expression in muscles 5 and 25 is also missing. (G) Wild-type stage 15 embryo stained with S59 intron probes and S59 antibodies and (H) stage 15 slou mutant embryo stained with S59 intron probe, which produces punctate signals of nascent nuclear transcripts. While both wild-type and slou mutant embryos show nascent S59 transcripts in the nuclei of the muscle 18 syncytia, the slou mutant lacks nascent transcripts at the positions of muscle 25 and in the majority of syncytia resembling muscle 27. (I) Wild-type stage 16 embryo carrying the RRHS59-lacZ reporter construct, probed with β-Gal and Nau antibodies. Muscle 27 expresses both β-Gal and Nau. (J) Stage 15 slou mutant embryo carrying RRHS59-lacZ, stained as in (I). Muscle 27 retains Nau expression. (K,L) Stage 14 embryos carrying an ap^U67 (ap/lacZ) chromosome, probed with β-Gal and Kr antibodies (and S59 antibodies in blue to identify homozygous mutants – not shown). The embryo with a wild-type background (K) shows coexpression of Kr and ap/lacZ in muscle 27, while the slou mutant embryo (L) is lacking ap-lacZ in this muscle (Note that the single β-Gal-containing nuclei near muscles 27 are probably associated with PNS; Cohen et al., 1992).
(S59) function is required for the normal specification and/or differentiation of muscle fibers which, in turn, is necessary for the late activation of S59 expression in some of the syncytia. This notion, which would allow but not require autoregulation, is consistent with our identification of separate early- and late-acting slou enhancer elements (S. K. and M. F., data not shown).

**Regulatory interactions between slou (S59) and lb in lateral somatic muscle development**

The observed loss of muscles 5 and 25 in slou (S59) mutant embryos, which are derived from two daughter cells of a common progenitor (cluster I, Dohrmann et al., 1990), is strictly correlated with the duplication of the segment border muscle (muscle 8) (Fig. 1E). Because muscle 8 comes from the same lateral areas as the progenitor of 5/25, this observation raises the question of whether loss of slou activity causes a transformation of the 5/25 progenitors/founders into muscle 8 progenitors/founders. Alternatively, slou mutation could have non-autonomous effects on neighboring myoblasts causing a duplication of muscle 8. Recently, it has been shown that the homeobox gene lb is expressed in muscle 8 and its progenitor and functions in specifying this muscle, as well as the lateral adult muscle precursor that is derived from a sibling founder cell (Jagla et al., 1998).

As a first step towards clarifying the developmental relationships between the S59- and lbexpressing lateral muscles, we performed a comparative expression analysis of the two genes in wild-type embryos. During early stage 11, after S59 expression has initiated in the 5/25 progenitor, lb expression is observed in a dorsally abutting cluster of 6-7 cells (Fig. 4A,B). Shortly thereafter, lb expression becomes restricted to two progenitors within this promuscular cluster, which are still closely associated with the S59-expressing progenitor (Fig. 4C,D). During late stage 11, the S59 progenitor divides asymmetrically to generate two founders, namely a larger, dorsally positioned founder 5 and a smaller, more ventral founder 25 (Fig. 4E-H). Upon completion of this division, each of the two lb progenitors also divides once to generate four founders that are arranged in close proximity (Fig. 4F-H). (Note that this description differs slightly from that given by Jagla et al. [1998], who reported that lb expression is restricted to a single progenitor that undergoes two divisions to generate a total of three founder cells.) During late stages 12-13, muscle 25 founder migrates ventrally along a diagonal path to form muscle 25 in the posteriorly adjacent segment, whereas the muscle 5 founder migrates slightly dorsally to form muscle 5 (Fig. 4L; Dohrmann et al., 1990). At the same time, one of the four lb founders migrates dorsally along the segment border and ultimately fuses with surrounding myoblasts to form muscle 8 (Fig. 4I-L). Two of the three remaining lb cells stay unfused at their original position and form two lateral adult precursor cells (Jagla et al., 1998). The third cell rapidly

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**Fig. 4.** Comparison of Slou (S59) and Ladybird expression in normal muscle development. Left and right hand panels show low- and high-magnification views, respectively, of the same or same stage embryos; anti S59 is shown in green (arrows) and anti Lb in red (arrowheads). (A,B) Early stage 11. S59 protein is seen in the 5/25 progenitor and Lb in all cells of the neighboring promuscular cluster. (C,D) Mid-stage 11. S59 is still seen in the 5/25 progenitor, whereas Lb expression is now restricted to two muscle progenitors. (E-H) Late stage 11. The S59 progenitor has divided into the muscle 5 and muscle 25 founder and the two Lb progenitors divide one after the other to yield four adjacent founders. (I,J) Late stage 12. The founders of muscles 5 and 25 have migrated apart and in between the two, the Lb-expressing founder of muscle 8 is in the process of fusing with surrounding myoblasts. LaPs. Two lateral adult muscle precursor cells expressing Lb. (K,L) Stage 15 showing the mature pattern of S59 and Lb expression. Muscle 5 (as well as 26 and 29) has ceased to express S59.
loses lb expression, which has prevented us from defining its ultimate fate.

To determine whether the duplication of muscle 8 in the absence of slou (S59) activity is due to ectopic expression of lb, we performed a similar analysis in slou mutant embryos. In this analysis, we used an S59-lacZ line that expresses lacZ in early stages in the progenitor and founder cells for muscle 5 and 25 to follow lb cells and S59 founders (see Materials and Methods). As shown in Fig. 5E,F (compare with Fig. 5A,B), slou mutants indeed display lb expression in five instead of three, and upon division of the second lb progenitor in six instead of four, founder cells. Inspection of S59/lacZ and lb doubly stained embryos clearly demonstrates that the ectopic lb-expressing cells correspond to the cells that are supposed to be muscle 5 and muscle 25 founders (Fig. 5E, compare with Fig. 5A). Furthermore, analysis of late-stage slou mutant embryos shows that the supernumerous lb founders now proceed along developmental pathways that are typical for bona fide lb cells. Thus, they form a second lb-expressing muscle 8 (Fig. 5G, compare with Fig. 5C) and an extra lateral adult muscle precursor (i.e., a total of three instead of two of them; Fig. 5H, compare with Fig. 5D) in each segment.

Because these observations suggested a normal function of slou (S59) in repressing lb in the founders of muscles 5 and 25, we tested whether ectopic expression of S59 could also repress lb in the authentic founders of muscle 8 and lateral adult muscle precursors. As shown in Fig. 5I,J, pan-mesodermal expression of S59 indeed causes a strong reduction in the number and, in many segments, a complete loss, of lb-expressing muscle founders. As a consequence, many segments are lacking muscle 8 and lateral adult precursors in later stages, thus contributing to the strong aberrations in the muscle patterns seen in these embryos (Fig. 5K).

In addition to muscles 5 and 25, we had also observed loss

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Fig. 5. Cell fate transformations upon mutation and ectopic expression of slou (S59). (A) Wild-type stage 11. S59 protein (green, arrows) and Lb protein expression (red, arrowhead) upon division of the 5/25 progenitor and of the first Lb progenitor. (B) Wild-type stage 12. Lb expression in four founders upon division of the second progenitor. Arrow, Lb expression in ventral epidermis. (C) Wild-type stage 16. Lb expression in one syncytium (muscle 8) per hemisegment (two Lb-expressing lateral adult muscle precursors associated with muscle 8 are not clearly discernible). (D) Wild-type stage 16. Twist antibody staining shows a pair of lateral adult precursors (LaPs) and one ventral adult precursor per hemisegment (arrows). DLaPs, three dorsolateral adult precursors; DaP, one dorsal adult precursor per hemisegment. Tracheal signals are unspecific. (E) Stage 11 slou mutant embryo carrying an RRRHS59-lacZ insertion, stained with β-Gal and Lb antibodies. Ectopic Lb is coexpressed with S59/lacZ in the cells that would normally become muscle 5 and muscle 25 founders (arrows). (F) Stage 12 slou mutant embryo showing Lb expression in six instead of the normal four founder cells (arrowhead). Arrow, see B. (G) slou mutant embryo stained as in C with duplicated Lb-stained muscle 8 in each segment. (H) slou mutant embryo with one additional lateral adult precursor (LaP) and one additional ventral adult precursor (VaP) in each hemisegment (arrows, compare with D). (I-L) Embryos with ectopic S59 expression driven by the GALSG30 driver (twiGAL4+24B GAL4). (I-K) Lb expression is severely reduced and in some hemisegments lost upon ectopic S59 expression (arrowheads and bracket; arrows denote Lb expression in ventral epidermis). (L) Twi-expressing lateral adult precursors (LaPs) and dorsolateral adult precursors (DLaPs) are frequently missing upon ectopic S59 expression (brackets).
of muscle 29 in slou mutants. Examination of the fate of the 29 muscle founder in the absence of slou activity reveals the appearance of a second ventral adult muscle precursor cell, a cell type that is normally formed by the sibling of the 29 founder (Fig. 5H compare with D; Carmena et al., 1995). Thus, in the absence of S59, it appears that the default fate of these two founders is that of ventral adult muscle precursors, and that S59 expression normally promotes a muscle 29 fate in one of them. This is consistent with the transiently higher levels of S59 expression in the muscle 29 founder as compared to the ventral adult muscle precursor (unpublished observations). However, the failure to suppress formation of ventral adult precursors upon ectopic S59 expression (Fig. 5L) indicates that slou (S59) is required, but not sufficient to specify one of the two siblings as muscle 29.

**Persistence and readjustments of muscle patterns during adult muscle morphogenesis**

Since some slou (S59) mutant animals escape lethality, we were able to study the consequences of slou mutation for adult muscle patterning. The ventral and lateral musculatures of three representative abdominal segments from wild-type and slou mutant flies are shown in Fig. 6A,B, respectively. Shortly after eclosion, the larval segment border muscles (muscles 8) in wild-type flies still persist, whereas other larval muscles have been histolyzed and replaced by the adult muscle groups derived from corresponding adult muscle precursors (Bate et al., 1991; Broadie and Bate, 1991). As predicted from the results described above, freshly eclosed slou mutant flies display a duplicated muscle 8 in every abdominal hemisegment (Fig. 6B, compare with Fig. 6A). Because slou mutant embryos were found to have one supernumerary ventral and lateral adult muscle precursor, one might expect to find corresponding increases in the number of ventral and lateral adult muscle fibers. However, we find that these muscle groups contain essentially the same numbers of muscle fibers as in wild-type flies (~6 ventral fibers and ~20 lateral fibers per abdominal hemisegment; Miller, 1950). This result indicates that there is not a strict one-to-one relationship between the number of embryonic muscle precursors and the corresponding adult muscle fibers and suggests that there are mechanisms to readjust the number of muscles. Alternatively, it is possible that the extra adult muscle precursors in slou mutants are not fully functional and unable to form adult muscle fibers.

slou mutant flies display sluggish movements, are unable to fly and the majority has outspread, upheld or drooping wing postures (Fig. 6D, compare with Fig. 6C). Because embryonic thoracic segments, which produce the flight muscles, contain a large number of S59-expressing myoblasts (Dohrmann et al., 1991), we sectioned adult slou mutant flies to determine whether these phenotypes might be due to abnormalities in flight muscle development. As shown in Fig. 6F (compare with Fig. 6E) slou mutant flies have a full complement of direct and indirect flight muscles, and their overall morphology closely resembles that of corresponding muscles from wild-type flies. However, it is possible that loss of slou activity causes more subtle defects in thoracic muscle differentiation. Because S59 is also expressed in specific neurons of the CNS, the observed motoric and wing posture phenotypes could also result from defects in the development and function of motoneurons, which were not analyzed in the present study.

**DISCUSSION**

The pattern of the larval somatic musculature of Drosophila is fairly complex and therefore it is not surprising that the generation of this stereotyped pattern requires intricate regulatory circuits. It has been proposed that a major role of
these regulatory inputs is to specify and diversify a distinct subpopulation of myoblasts, the muscle founder cells, and to provide them with the necessary information to form the mature muscle pattern. The data from the present study provide new support for the notion that, in molecular terms, these regulatory inputs lead to the expression of specific combinations of identity genes in founder cells, many of which encode transcription factors that go on to execute specific developmental programs. In addition, we are now beginning to understand some of the details of the regulatory hierarchies in muscle patterning.

The qualitatively distinct effects of slou (S59) mutation for the development of different muscle groups are presumably due to the presence of certain other transcription factors and their functional importance relative to slou in the respective founder cells. Our data also suggest that slou functions in different muscle progenitors and founders at different levels of the regulatory hierarchy, which is reflected in the differential effects seen in slou mutants. In this context, it is worth noting that the impact of slou mutation, which is strongest with respect to muscles 5 and 25, intermediate with respect to muscles 26/27, and weakest with respect to muscles 11/18, correlates well with the temporal onset of slou (S59) expression in wild-type embryos. Initial S59 expression is observed in the progenitor of the muscles 5/25 (cluster I), then in the progenitors of muscles 26/27/19/VaP (cluster II), and finally in the progenitors of muscles 11/18 (cluster III) (Dohrmann et al., 1990).

Within cluster II, Slou (S59) is expressed and appears to function in combination with at least four other transcription factors, Nautilus, Apterous, Krüppel and Msh (Paterson et al., 1991; Bourgouin et al., 1992; Ruiz-Gomez et al., 1997; Nose et al., 1998). As with S59, the expression of Kr, Nau and Ap persists in the respective founder and muscle 27. Moreover, Kr has been shown to function as an upstream regulator of S59 expression. While nau and ap do not appear to be required for normal muscle 27 development, possibly due to functional redundancy with Kr, slou and yet unknown genes, the absence of Kr activity leads to a transformation of muscle 27 identity into that of its sibling, muscle 26 (Bourgouin et al., 1992; Ruiz-Gomez et al., 1997; Keller et al., 1998). Because slou (S59) expression in muscle 27 founders is lost in the Kr mutant background, the observed transformation is presumably due to the absence of both Kr and slou activity and, possibly, additional regulators that depend on these factors.

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**Fig. 7.** Schematic summary and proposed regulatory interactions during successive stages of lateral muscle development in wild-type and slou mutant embryos. By repressing lb, slou (S59) switches two lateral muscle founders from a default fate as muscle 8/LaP founders to their proper fate as muscle 5 and muscle 25 founders (see text). (A-C) Proposed regulatory inputs that activate broad lb expression (A), progenitor-specific slou (S59) expression (B), and progenitor-specific lb expression (C) within two neighboring promuscular clusters. Green denotes cells that express/contain S59 mRNA and ‘S59’ cells that contain functional S59 protein. Yellow/red cells are labeled analogously for lb expression and function. Mutant and ectopic expression analysis indicate that numb is required during the asymmetric division of the 5/25 progenitor to generate muscle 5 founder identities (S. K. and M. F., unpublished data).
Kr. This view is consistent with our observation that slou mutation does not cause a complete transformation of muscle 27 into muscle 26, but rather a phenotype that can be interpreted as partial transformation. Thus, during development of muscle 27, Kr acts upstream of slou (S59) to maintain its expression, whereas slou is not required for the maintenance of Kr. Based upon the observed phenotypes, we propose that, in addition to slou, Kr has several other targets that are important for muscle 27 development, and only the simultaneous absence of these regulators would cause a complete muscle 27 to muscle 26 transformation. Alternatively, Kr may be required in parallel with slou (S59) and other target genes during muscle 27 differentiation. Surprisingly, morphological changes are only observed in ~70% of muscles 27 in Kr mutant embryos (Ruiz-Gomez et al., 1997), whereas S59 expression fails to be maintained in 100% of them. Because we observe muscle 27 abnormalities in 100% of slou mutants, this would suggest that the early, Kr-independent expression of identity genes such as slou (S59) in muscle 27 founders is in many cases sufficient to produce a normal muscle.

In muscle 29, Ap and Msh, but not Kr are coexpressed with Slou (S59). Whereas ap mutation produces only occasional loss of muscle 29 (Bourgouin et al., 1992), the absence of slou activity results in the transformation of 100% of these muscles into their siblings, the ventral adult muscle precursors (VaPs). Thus, slou may have a role in the founder of muscle 29 that is functionally equivalent to that of Kr in the muscle 27 founder, as discussed above.

Our observations with respect to cluster I provide novel clues about the regulatory pathways from promuscular clusters to identified muscles. We show that muscle progenitors from neighboring clusters segregate at different time points in early mesoderm development. Specifically, at the time when lb expression initiates in the entire promuscular 8/LaP cluster, the muscle 5/25 progenitor of the adjacent cluster is already specified and expresses S59. We propose that this is due to sequential regulatory inputs received by these cell clusters (Fig. 7). An initial input (‘A’ in Fig. 7) may serve to activate lb at intermediate levels in all cells of the promuscular cluster, and perhaps at the same time define this cluster whereas another input (‘B’), which is received by the neighboring cluster, triggers S59 expression in the 5/25 progenitor in a process that also involves lateral inhibition through Notch signaling (Bate et al., 1993; Baker and Schubiger, 1996). In a subsequent step, a functionally analogous input (‘C’) would then serve to restrict lb expression to two progenitors in the promuscular 8/LaP cluster. Interestingly, based upon our observations in slou mutants, input C appears not to be restricted to this cluster, but may cover both clusters or at least include the areas where they abut. The reason for the exclusive response of the 8/LaP cluster to this input is that slou (S59) and active Notch (Jagla et al., 1998) apparently repress lb activation by input C in the 5/25 cluster. This model would explain why, in the absence of slou activity, the cell normally destined to become a 5/25 progenitor, (in which the Notch pathway is inactive) expresses lb ectopically. The final effects on the muscle pattern are very similar to those obtained with GAL4/UAS-driven ectopic lb expression (Jagla et al., 1998). The model is also in agreement with our data showing that ectopic expression of S59 can repress lb in the 8/LaP cluster, producing muscle phenotypes similar to those of lb loss of function. An additional requirement for a response to these spatially localized inputs, which is shared by the cells in both clusters, is the activity of tinman, which may act as a mesoderm-specific coactivator for slou (S59) and lb activation by external inputs (Azpiazu and Frasch, 1993; Jagla et al., 1998).

What might be the nature of these sequential inputs? The observation of the occurrence of sequential events during promuscular cluster/muscle progenitor formation is reminiscent of previous observations made in the dorsal somatic mesoderm. In these studies, it was shown that an initial, FGF-receptor (Htl)-dependent signal is involved in delineating a promuscular cluster, C2, and in specifying a progenitor within it, which in turn gives rise to a pair of eve-expressing pericardial cells. A subsequent signal, which is mediated by the EGF-receptor (DER/Flb, perhaps together with Htl), functions analogously to define an adjacent cluster, C15, and an eve-expressing progenitor within it that gives rise to muscle 1 (Buff et al., 1998; Carmena et al., 1998a; Michelson et al., 1998). Whereas the Htl-activating signal has not been identified, these authors have suggested that localized Rhomboid expression in the initially formed progenitor P2, in conjunction with the more broadly expressed ligands Spitz and Vein, may trigger DER-activation in the adjacent C15 cluster and P15 progenitor (see also Yarnitzky et al., 1998). It is possible that promuscular cluster formation and progenitor specification in the lateral somatic mesoderm follows a similar sequence of events. Since embryos with reduced Htl activity appear to lack muscles 5, 8 and 25 and embryos with reduced DER activity lack muscle 8, but not muscles 5 and 25 (Buff et al., 1998; Michelson et al., 1998), it is conceivable that input ‘B’ (Fig.7) activates S59 through Htl, whereas input ‘C’ activates lb through DER (in the absence of S59). We do not know whether the progenitor of muscles 5/25 expresses Rhomboid or another DER-activating signal but, if so, our data show that slou is not required for activating the expression of such signals. Rather, slou functions in a strictly cell-autonomous manner, since its mutation affects only muscles that are derived from slou (S59)-expressing progenitor and founder cells. Mechanistically, one of these functions is likely to include direct repression of lb transcription in the progenitor of muscles 5/25.

Besides its involvement in early cell fate decisions, it is plausible that slou continues to function in the developing muscles that maintain its expression. Of note, our in situ hybridization experiments with slou (S59) intron probes show that upon division of the progenitor 5/25, the muscle 5 founder maintains transcription until fusion, whereas the muscle 25 founder initially stops transcribing slou (S59) but resumes expression upon fusion with myoblasts at its final position. In addition, the nuclei of newly fused cells also initiate slou (S59) transcription, thus providing high levels of Slou (S59) protein in muscles 18, 25 and 27 during their later stages of development. Although we do not have any direct evidence, this intricate regulation suggests that the differential maintenance of slou (S59) could be directly involved in the control of certain late aspects of muscle development, such as muscle differentiation and morphogenesis, and possibly also innervation.

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